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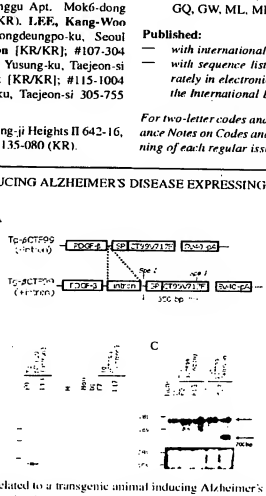
(54) Title: TRANSGENIC MICE INDUCING ALZHEIMER'S DISEASE EXPRESSING MUTANT BETACTF99



(57) Abstract: The present invention is related to a transgenic animal inducing Alzheimer's disease. More particularly, the present invention is a vector for transformation of animal comprising a carboxyl-terminla fragments of mutant human beta amyloid protein which contains Indiana mutation (βCTF99) and a transgenic mouse inducing Alzheimer's disease prepared by microinjection of the same into a pronuclei of a fertilized oocyte. The transgenic mouse of the present invention exhibited clinical symptoms of Alzheimer's disease such as decreases of cognitive ability and memory, and increases of anxiety. Therefore, the transgenic mouse of the present invention will be a useful animal model for a research of Alzheimer's disease. Particularly, since the transgenic mouse of the present invention showed more remarkable decreases of cognitive ability than any other transgenic animal model for Alzheimer's disease known in the art, the transgenic mouse of the present invention can be used as an animal model for disease relating anxiety.

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[DESCRIPTION]

[Invention Title]

TRANSGENIC MICE INDUCING ALZHEIMER'S DISEASE EXPRESSING MUTANT BETACTF99

[Technical Field]

The present invention relates to a transgenic mouse with induced Alzheimer's disease pathology, more precisely, a transgenic mouse that shows Alzheimer's disease pathology induced by the insertion of a cDNA of a mutant human amyloid beta precursor protein into chromosomal DNA.

[Background Art]

Increased production of β-amyloid peptide (referred "Aβ" hereinafter) has reported to be involved in the pathogenesis of Alzheimer's disease (AD). Aβ can be produced by sequential action of β-secretase and γ-secretase inducing proteolytic cleavages of APP. Presenilin-1 (referred "PS1" hereinafter) may be a key component of γ-secretase complex or regulate traffics of its' matrix (Esler WP and Wolfe MS, 2001, *Science*, 293:1449-1454). Thus, PS1 has been considered to be a therapeutic target for the treatment of AD and the delayed expression of AD symptoms (Esler WP and Wolfe MS, 2001, *Science*, 293:1449-1454; Li YM *et al.*, 2000, *Nature*,

405:689-694). However, it is still in doubt whether or not γ-secretase inhibitor activity is involved in the accumulation of β-CTF.

Recent studies with a conditional knockout strategy have circumvented the lethality of PS1 deficient mice and generated adult animals lacking PS1 specifically in the brain (Yu H *et al.*, 2001, *Neuron*, 31:713-726; Dewachter I *et al.*, 2002, *J. Neurosci*, 22:3445-3453). The study invclving these double transgenic mice carrying both the PS1 conditional mutation and the APP_{V717I} transgene revealed that the elimination of the γ-secretase activity provided by PS1 markedly reduced Aβ production, plaque deposition and rescued impaired hippocampal LTP, but that it neither corrected the deficits in memory that APP_{V717I} transgenic mice displayed nor stopped the progress of neurophysiological and pathogenic symptoms (Dewachter I *et al.*, 2002, *J. Neurosci*, 22:3445-3453). Although the underlying mechanism has not yet been clearly elucidated, these studies show that the loss of γ-secretase activity in the brain leads to the severe accumulation of beta-CTF99, and raise the possibility that βCTF99 accumulation might cause cognitive deficits in the absence of plaque deposition in their double transgenic mice. It also raises the question as to whether or not other biochemical

impairments or behavioral alterations present in APP_{V717I} transgenic mice can be reverted in their double transgenic mice. Regarding that PS1 has pleiotropic roles in brain cell functions including Norch (Naruse S *et al.*, 1998, *Neuron*, 21:1213-1221; Song W *et al.*, 1999, *Proc. Natl. Acad. Sci. USA.*, 96:6959-6953) and N-cadherin processing (Marambaud P *et al.*, 2003, *Cell*, 114:635-645), it needs to be answered whether or not the observed memory deficits of these double transgenic knockout mice were produced solely by βCTF99.

More direct evidence for the *in vivo* role of βCTF99 is to be ascertained from studies with transgenic mice expressing βCTF99 in the brain. Eight research groups have independently created transgenic mouse lines expressing various CTF forms of the human APP in the brain. Four of those lines showed either neuronal atrophy (Oster-Granite *et al.*, 1996, *J. Neurosci.*, 16:6732-6741; Nalbantoglu J *et al.*, 1997, *Science*, 387:500-505; Sato et al., 1997, Dement Geriatr Cogn Disord, 8:296-307) or impaired learning (Nalbantoglu J *et al.*, 1997, *Science*, 387:500-505; Berger-Sweeney J *et al.*, 1999, *Brain Res Mol Brain Res*, 66:150-162; Laronde R *et al.*, 2002, *Brain Res*, 956:36-44) at age 12 - 28 months, whereas the other four lines did not display any obvious neuronal loss or

cognitive impairment (Sandhu *et al.*, 1991, *J Biol Chem.*, 266:21331-21334; Araki *et al.*, 1994, *Int. J. Exp. Clin. Invest.*, 2:100-106; Sberna *et al.*, 1998, *J. Neurochem.*, 71:723-731; Li *et al.*, 1999, *J. Neurochem.*, 72:2479-2487; Rutten *et al.*, 2003, *Neurobiol Dis ., 12: 110-120*). Thus, the developed transgenic mice expressing CTFs showed conflicting results that raged from no phenotype to AD-like pathogenesis. Accordingly, the *in vivo* role of βCTF99 remains elusive.

The present inventors have thus tried to establish an animal model for AD study, and finally prepared a transgenic mouse line bearing clinical symptoms and characteristics of AD. And further, the inventors have completed the present invention by confirming that this newly created transgenic mouse clearly shows AD symptoms.

【Disclosure】

【Technical Problem】

The object of the present invention is to provide a transgenic vector that can be used to create transgenic mice showing AD pathology.

Another object of the present invention is to create a genetically stable transgenic mouse carrying the above vector.

[Technical Solution]

To achieve the above objects, the present invention provides a transgemic vector that contains a gene coding a C-terminal fragment (CTF) of mutant human amyloid beta precursor protein (APP).

The present invention also provides a transgenic mouse that was produced by injection of the vector into the nucleus of a fertilized egg of mice, followed by transferring injected eggs into the oviduct of foster mothers to generate mice.

[Advantageous Effects]

The transgenic mouse of the present invention showed remarkable cognitive impairments compared to those of the wild type mouse both in the Morris water maze test and in passive avoidance test. In addition, the transgenic mouse showed highly increased anxiety compared to that of the wild type mouse in the elevated plus maze test, indicating that this AD animal presents AD symptoms more clearly than any other known AD animal models. Since the transgenic mouse of the present invention shows clear AD symptoms, this animal can be used as an animal model not only for studies of AD pathogenesis but also for studies on cognitive and anxiety impairments.

【Description of Drawings】

Fig. 1A is a schematic diagram showing the vectors 'PDGF-β CTF99(V717F)-pA' and 'PDGF-intron-β CTF99(V717F)-pA' for transformation constructed in the present invention.

Fig. 1B is a photograph of Southern blotting confirming the insertion of βCTF99(V717F) mutant gene in transgenic animals of the invented 'Tg-βCTF99/B6(-intron)' and 'Tg-βCTF99/B6(+intron)'. In the photograph, the arrow presents 350 bp βCTF99 fragment digested by *Spe*I.

Fig. 1C is a photograph of Northern blotting confirming the expression of βCTF99(V717F) mutant gene in transgenic mice of the invented 'Tg-βCTF99/B6(-intron)' and 'Tg-βCTF99/B6(+intron)'. In the photograph, the upper arrow presents internal βCTF99 transcript (3.5 kb) and the lower arrow presents mutant βCTF99 transcript (700 pb) of the present invention.

Fig. 2A is a set of a photograph of Western blotting confirming the production of βCTF99 protein in Tg-βCTF99/B6 transgenic mice of the present invention (left

panel) and a graph showing the quantification of the production above (right panel). In the photograph of Western blotting, the upper and lower panels are prepared using αCTF antibody and βCTF antibody, respectively. And in the graph, the data obtained from four different experimental groups are presented as the means ± SEM.

Fig. 2B-C are photographs of immunohistological analysis investigating the expression of βCTF protein in cerebral cortex (CX) of Tg-βCTF99/B6 transgenic mouse (C) of the present invention and their wild type mice(B).

Fig. 3A is a photograph of Western blotting measuring the expressions of p-JNK, p-c-Jun, JNK1, JNK2, JNK3, p-ERK, ERK, p-p38 and p38α protein in the brain of Tg-βCTF99/B6 transgenic mouse of the present invention.

Fig. 3B is a graph showing the expression levels of p-JNK (left panel) and p-c-Jun (right panel) in the brain of Tg-βCTF99/B6 transgenic mice of the present invention. Data presented are the means ± SEM of 7 and 4 independent experiments on 6 and 4 animals (n=4-6) for p-JNK and p-c-Jun, respectively.

Fig. 4A is a set of a photograph (left panel) of

Western blotting measuring the expressions of Bcl-2, Bcl-x_L, Bad and Bax proteins in the brain of Tg-βCTF99/B6 transgenic mouse of the present invention at 14-15 months and a graph (right panel) is the results above presented in relative expression levels. Each data obtained from 3 other experimental groups is presented as the means ± SEM.

Fig. 4B is a photograph resulted from immunohistological analysis of the expressions of Bad and Bax proteins in CA1, CA3 and DG regions of hippocampus (HP) in the brain of Tg-βCTF99/B6 transgenic mouse at 14-15 months of the present invention, and a graph (right panel) is the results above presented in relative expression levels. In the photograph, the scale bar in the upper panel represents 200 ㎛ and three scale bars in the lower panel represent 500 ㎛ each.

Fig. 5A is a photograph (left panel) of Western blotting measuring the expression of calbindin protein in the brain of Tg-βCTF99/B6 transgenic mouse at 15 months of the present invention and a graph (right panel) is the results above presented in relative expression levels. Each data obtained from three independent experimental groups is presented as the means ± SEM.

Fig. 5B is a photograph of immunohistological analysis measuring the expression of calbindin protein in CA1, CA3 and DG region of hippocampus (HP) in the brain of Tg-βCTF99/B6 transgenic mouse at 15 months of the present invention, and a graph (right panel) is the results above presented in relative expression levels. The scale bar in the upper panel represents 200 μm and the scale bars in the lower panel represent 500 μm each.

Fig. 6A is a set of a photograph (left panel) of Western blotting measuring the expressions of CREB and phosphorylated-CREB proteins in the brain of Tg-βCTF99/B6 transgenic mouse at 15 months of the present invention and a graph (right panel) is the results above presented in relative expression levels. In the graph, each data obtained from three independent experimental groups is presented as the means ± SEM.

Fig. 6B-K are photographs of immunohistological analysis measuring the expressions of CREB and phosphorylated-CREB proteins in CA1 of hippocampus (HP), cerebral cortex (CX) and DG regions in the brain of Tg-βCTF99/B6 transgenic mouse at 15 months of the present invention, and a graph (right panel) is the results above presented in relative expression levels. The scale bars

in panel C, E and K represent 50 ㎛ each.

Fig. 7A-H are photographs of immunohistological analysis measuring the expressions of Neu-N protein (A-D) and MAP2 protein (E-H) in CA1 region of hippocampus (HP) and cerebral cortex (CX) of the brain of Tg-βCTF99/B6 transgenic mouse of the present invention at 18 months and the wild type control mouse.

A: Prefrontal cortex of the wild type control mouse was stained with anti-Neu-N antibody,

B: Prefrontal cortex of Tg-βCTF99/B6 transgenic mouse was stained with anti-Neu-N antibody,

C: Pyramidal cells of the wild type control mouse were stained with anti-Neu-N antibody,

D: Pyramidal cells of Tg-βCTF99/B6 transgenic mouse were stained with anti-Neu-N antibody,

E: Prefrontal cortex of the wild type control mouse was stained with anti-MAP2 antibody,

F: Prefrontal cortex of Tg-βCTF99/B6 transgenic mouse was stained with anti-MAP2 antibody,

G: CA1 of the wild type control mouse was stained with anti-MAP2 antibody,

H: CA1 of Tg-βCTF99/B6 transgenic mouse was stained with anti-MAP2 antibody.

Fig. 7I is the result showing gradual neurodegeneration revealed by measuring the expression of Neu-N protein in the brains of Tg-βCTF99/B6 transgenic mice at 12 and at 18 months of the present invention.

Fig. 8A is the result of the open field test showing the locomotor activities of Tg-βCTF99/B6 transgenic mice at 7 and at 14 months of the present invention.

Fig. 8B is the result of the rota-rod test showing the locomotor activities of Tg-βCTF99/B6 transgenic mice at 5.5 and at 11 months of the present invention. In the graph, the data obtained from 6-15 independent experimental groups are shown as the means ± SEM.

Fig. 9A-B is the result of Morris water maze test. The latency to find a hidden platform was recorded to investigate cognitive impairments of Tg-βCTF99/B6 transgenic mice at 7 months of age (A) and at 14 months of age (B) of the present invention. In the graph, * indicates a difference at the $p<0.05$ level in each group (Student's t-test). The data obtained from 6-8 independent experimental groups are presented as the means ± SEM.

Fig. 9C shows the results of the Morris water maze

test showing swimming speed of animals to find a hidden platform, which was investigated to measure whether the transgenic animals of the present invention have any general motor function impairments. In the graph, * indicates a difference at the $p<0.05$ level in each group (Student's t-test). The data obtained from 6-8 independent experimental groups are presented as the means ± SEM.

Fig. 9D shows the results of the passive avoidance test to investigate cognitive impairments of Tg-βCTF99/B6 mice at 7 months and at 14 months of the present invention. In the graph, * indicates a difference at the $p<0.05$ level in each group (Student's t-test). The data obtained from 6-8 independent experimental groups are presented as the means ± SEM.

Fig. 10 shows the results of the elevated plus maze test to investigate anxiety state of Tg-β CTF99/B6 mice at 13 months of age of the present invention. In the graph, * indicates a difference at the $p<0.05$ level in each group (Student's t-test). The data obtained from 7-10 independent experimental groups are presented as the means ± SEM.

【Best Mode】

Hereinafter, the present invention is described in detail.

The present invention provides a transgemic vector that contains a gene coding a C-terminal fragment of mutant human amyloid beta precursor protein (APP), which can be used in the generation of AD mouse model.

The above C-terminal fragment of mutant human amyloid beta precursor protein (APP) includes the C-terminal fragment of APP bearing V717F mutation, which was produced by the replacement of valine (V) with phenylalanine (F), which is prepresented by SEQ. ID. No 1. That is, the C-terminal fragment of APP bearing V717F mutation is preferred to have an amino acid sequence represented by SEQ. ID. No 3. In the preferred embodiment of the present invention, the mutant βCTF99 represented by SEQ. ID. No 3, which was then named "βCTF99(V717F)", was prepared by PCR using the second half of APP_{V717F} cDNA represented by SEQ. ID. No 2 as a template.

It is also preferred for the transgenic vector of the present invention, which includes PDGF-β promoter, mutant βCTF99(V717F) encoding an amino acid sequence represented by SEQ. ID. No 3, and SV40 polyadenylation sequence. To increase translation efficiency, Kozac sequence was introduced in front of the above mutant βCTF99(V717F). The vector of the present invention was

designed to include PDGF-β promoter, Kozac sequence, mutant βCTF99(V717F) represented by SEQ. ID. No 3 (βCTF99(V717F)) and SV40 polyadenylation sequence. The resulting vector was then named "PDGF-βCTF99(V717F)-polyA" (see Fig. 1).

It is also preferred to construct the transgenic vector of the present invention, which has the intron B of the human β-globin gene inserted between PDGF-β promoter and βCTF99(V717F). The introduction of the intron B gene of the human β-globin gene is to increase expression efficiency of the βCTF99(V717F) gene and transcription stability. So, in the present invention, the transgenic vector was constructed to include the PDGF-β promoter, intron B of the human β-globin gene, Kozac sequence, mutant gene coding an amino acid sequence represented by SEQ. ID. No 3 (βCTF99(V717F)) and SV40 polyadenylation sequence. The resulting vector was named "PDGF-intron-βCTF99(V717F)-polyA" (see Fig. 1A).

The present invention also provides a transgenic mouse with induced Alzheimer's disease prepared by inserting the vector of the invention into a mouse chromosome.

PDGF-βCTF99(V717F)-polyA or PDGF-intron-βCTF(V717F)-polyA transgenic vector is preferably

introduced into the pronucleus of mice to produce a transgenic mouse of the present invention, and PDGF-intron-βCTF99(V717F)-polyA is more preferred. In the preferred embodiment of the present invention, PDGF-βCTF99(V717F)-polyA or PDGF-intron-βCTF99(V717F)-polyA transgenic vector was microinjected into the pronuclei of fertilized eggs prepared from inbred C75BL/6 mice, and the injected eggs were transplanted in surrogate mice. Comparison in expressions of βCTF99(V717F) mutant gene among the second generation produced from the surrogate mice and also offsprings produced by inbred was made. As a result, the expression of the mutant gene was much higher in transgenic mice transformed with PDGF-intron-βCTF99(V717F)-polyA vector than in other transgenic mice transformed with the other vector. The result indicates that it is preferred to transform a mouse by the introduction of PDGF-intron-βCTF99(V717F)-polyA vector to increase the insertion and expression efficiency of βCTF99(V717F) mutant gene of the present invention.

In the present invention, a transgenic mouse prepared by introducing PDGF-intron-βCTF99(V717F)-polyA vector for transformation into nucleus of a fertilized egg was named "Tg-βCTF/B6". After confirming that βCTF mutant gene was successfully inserted into a mouse and so βCTF protein was expressed to the wanted level therein,

the present inventors deposited the transgenic mouse of the invention at Korean Collection for Type Cultures (KCTC) of Korea Research Institute of Bioscience and Biotechnology (KRIBB) on March 10, 2003 (Accession No: KCTC 10609BP).

Gradual and age-dependent decrease of the expressions of calbindin and phosphorylated-CREB protein in hippocampus of transgenic mice with Alzheimer's disease induced in them(Tg-βCTF/B6) were observed. In the meantime, neurodegeneration, motor coordination deficit, cognitive deficits and anxiety, which are characteristics shown in the brain of human AD patients, were increased.

In the preferred embodiment of the present invention, calbindin expression was significantly reduced in the hippocampus of the brain of Tg-βCTF/B6 mice at 14-15 months of age (see Fig. 5). Calbindin is one of key components of calcium-binding proteins in the brain, along with parvalbumin and calretinin, which is presented as GABAergin and pyramidal neurons in various brain regions including frontal, temporal, entorhinal and hippocampus (Mikkonen *et al.*, 1999, *Neuroscience*, 92:515-532). Calcium-binding proteins regulate intracellular calcium concentrations due to their calcium buffering capacity. Altered intracellular calcium homeostasis may impair

normal cellular function and potentiate the cytotoxicity of neural cells (Berridge *et al.*, 1998, *Neuron*, 21:13-26; Mattson, MP, 1998, *Trends Neurosci*, 21:53-57; Carafoli, E., 2002, *Proc. Natl. Acad. Sci USA*, 99:1115-1122). Mice lacking of calbindin showed impairments in spatial learning and LTP (Molinari S *et al.*, 1996., *Proc. Natl. Acad. Sci USA*, 93:8028-8033). In aging or neurodegenerative brains, calbindin expression was reduced, leading to the pathologic changes (Iacopino A *et al.*, 1990, *Proc. Natl. Acad. Sci USA*, 87:4078-4082; Leuba *et al.*, 1998, *Exp Neurol.*, 152:278-291; Bu, J. *et al.*, 2003, *Exp Neurol.*, 182:220-231). Although numbers of AD models have been developed as of today, no explanation has been given on the co-relation between the decrease of calbindin and the decrease of cognitive function except the recent report on transgenic mice expressing human APP mutation (Palop *et al.*, 2003, *J. Neurosci.*, 100:9572-9577). And the present inventors have confirmed that the decrease of calcium-binding proteins is one reason for the cognitive deficits and other deficits that are characteristic features of AD patients.

The present inventors also observed the decrease of phosphorylated-CREB expression in hippocampus region of Tg-βCTF/B6 mice (Fig. 6). Antisense oligodeoxynucleotide-mediated disruption of the CREB gene

in the hippocampus was found to impair long-term memory formation (Guzoski *et al.*, 1997, *Proc. Natl. Acad. Sci USA*, 94:2693-2698), and a targeted mutation of the CREBβ isoform was associated with abnormal learning and memory (Bourechuladze *et al.*, 1994, *Cell*, 79:59-68; Blendy *et al.*, 1996, *EMBO J.*, 15:1098-1106). On the other hand, increasing the level of CREB in the brain enhanced the formation of long-term memory (Josselyn *et al.*, 2001, *J. Neurosci.*, 21:2404-2412). The decrease of phosphorylated-CREB expression in transgenic mice of the present invention resembles the result of investigation with AD patients. And, the reduced level of phosphorylated-CREB expression was confirmed to induce age-dependent cognitive impairment, neurodegeneration and the elevated anxiety. The above results indicate that transgenic mice of the present invention can serve as a useful AD model.

Tg-βCTF/B6 mice of the present invention showed similarities with the Tg2576+PS1P246L double transgenic mouse model (Savage *et al.*, 2002, *J. Neurosci.*, 22:3376-3385), as both AD models showed increased JNK activation (see Fig. 3), a feature displayed by the human AD brain (Zhu *et al.*, 2001, *J. Neurochem.*, 76:435-441; Savage *et al.*, 2002, *J. Neurosci.*, 22:3376-3385). Single transgenic mouse Tg2576 or Tg-PS1P246L did not show any changes in phosphorylated-JNK activation (Savage *et al.*, 2002, *J.*

Neurosci., 22:3376-3385), whereas transgenic mice of the present invention showed altered phosphorylated-JNK activation. In addition, transgenic mice of the present invention showed altered Bcl-2 family protein expressions in the brain (see Fig. 4). The expressions of Bcl-2, Bad and Bax proteins were significantly increased, whereas Bcl-x_L protein expression was reduced in transgenic mice of the present invention, indicating unbalanced Bcl-2 family protein expressions in the brain. The features of Bcl-2 family protein expressions in transgenic mice of the present invention were similar to those in human AD patients (Nagy *et al.*, 1997, *Neurobiol Aging*, 18:565-571; Kitamura *et al.*, 1998, *Brain Res.*, 780:260-269), suggesting that the transgenic mice of the present invention are very useful as an AD model.

The transgenic mice of the present invention (Tg-β CTF/B6) showed motor coordination deficit, cognitive deficits and increased anxiety, which are characteristics shown in AD patients (see Fig. 8 ~ Fig. 10). In the preferred embodiment of the present invention, in order to confirm whether or not Tg-β CTF/B6 mice showed clinical symptoms of AD, open field test, rota-rod test, Morris water maze test and passive avoidance test were performed to investigate cognitive capacity, and elevated plus maze test was performed to investigate anxiety. As a result,

in open field test, there was no significant difference in locomotor activity between wild type and transgenic mice (see Fig. 8A). In the meantime, in rota rod test, locomotor activity of transgenic mice was a little reduced, compared to that of the wild type mice, although the difference was not significant (see Fig. 8B). In Morris water maze test, transgenic mice showed cognitive deficits, resulting in impairment of memory (see Figs. 9A-C). In passive avoidance test, transgenic mice showed impairment of memory retention, compared to that of the wild type mice (see Fig. 9D). Besides, in elevated plus maze test, notably increased anxiety was observed in transgenic mice, compared to that of the wild type mice (see Fig. 10). Thus, the transgenic mice of the present invention can be effectively used as AD models because, as described above, they showed characteristic symptoms of AD such as memory deficits, cognitive deficits and increased anxiety.

[Mode for Invention]

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make

modifications and improvements within the spirit and scope of the present invention.

<Example 1> Preparation of human amyloid beta precursor protein cDNA and βCTF99 mutant gene

<1-1> Preparation of human amyloid beta precursor protein cDNA

The cDNA coding human amyloid beta precursor protein (referred 'APP' hereinafter) was prepared by PCR using Marathon-Ready cDNA library (Clontech, Palo Alto, CA, USA) constructed from human brain. The cDNA could not be amplified at once because the size of its open reading frame (ORF) was about 2.3 kb, taking APP770 as standard. Thus, the first half of the cDNA was amplified by using primer sets of app-1f primer represented by SEQ. ID. No 6 (5'-gcaagggtcgcgatgctgcccggtttg-3', the underlined part presented *Nru* I restriction enzyme recognition site) and app-2r primer represented by SEQ. ID. No 9 (5'-gacattctctctcggtgcttggcc-3'), and the resulting product was digested with *Nru* I and *Xho* I. The product was inserted into *Sma* I and *Xho* I restriction enzyme recognition sites of pBluescript II KS vector (Stratagene, USA). The second half of the cDNA was amplified by using primer sets of app-2f primer represented by SEQ. ID. No 8 (5'-cctacaacagcagccagtacccctg-3') and app-1r primer

represented by SEQ. ID. No 7 (5'-ggggg<u>actagt</u>tctgcatctgctc-3', the underlined part presented *Spe* I restriction enzyme recognition site), followed by digesting with *Spe* I and *Xho* I. Then, the resulting products were inserted into *Spe* I and *Xho* I restriction enzyme recognition sites of pBluescript II KS vector. The DNA fragments produced by digesting pBluescript II KS vector bearing the first half of the cDNA with *BamH* I and *Xho* I and the other DNA fragments produced by digesting pBluescript II KS vector bearing the second half of the cDNA with *Xba* I and *Xho* I were fused together with pBluescript II KS vector predigested with *Xba* I and *BamH* I, leading to the preparation of a vector construct carrying the full length of APP cDNA. In the meantime, three different isoforms were produced, according to the numbers of amino acid residues of coded protein, from the same gene of human beta amyloid by selective splicing. So, according to the numbers of amino acid residues, APP cDNA carries three different isoforms, that is, APP770, APP751 and APP695. The DNA sequences of the cloned cDNA were analyzed. As a result, among those three isoforms, the cloned cDNA was confirmed to be APP751 cDNA represented by SEQ. ID. No 1 coding APP751 (represented by SEQ. ID. No 2).

<1-2> Preparation of APP751 mutant gene

"V717F mutation (mutation in APP770 isoform induced by the replacement of valine, the 717th amino acid, with phenylalanine)" was introduced into APP751 cDNA by PCR. Particularly, PCR was performed by using pBluescript II KS vector carrying the second half of the APP751 cDNA produced in the above <Example 1-1> as a template with primer sets of app-2f primer represented by SEQ. ID. No 8 and app-717-r primer represented by SEQ. ID. No 11 (5'-caaggtgatgaagatcactgtcgc-3') with the 32 cycles of denaturation at 95℃ for 1 minute, primer annealing at 57℃ for 40 seconds and extension at 72℃ for 1 minute. And the resulting product was used for another PCR under the same conditions as described above by using primer sets of app717-f primer represented by SEQ. ID. No 12 (5'-gcgacagtgatcttcatcaccttg-3') and app-1r primer represented by SEQ. ID. No 7 this time. The PCR products from the two PCR above had "V171F mutation". So, the two PCR products were separated, slowly cooled down, extended with Klenow enzyme and then fused into one fragment. The fragment was digested with *Xho* I and *Spe* I by taking advantage of *Xho* I restriction enzyme recognition site of app-2f primer and *Spe* I restriction enzyme recognition site of app-1r primer, which were inserted into pBluescript II KS vector which was also digested with *Xho* I and *Spe* I ahead of time and the second half of APP cDNA was inserted in, leading to

the preparation of the mutant second half APP751 cDNA. In the meantime, the mutated DNA fragment prepared above was used to replace the corresponding region of pBluescript II KS vector where the full length of APP751 cDNA was inserted, resulting in APP751 mutant cDNA represented by SEQ. ID. No 3, which encodes a protein represented by SEQ. ID. No 4, and then named "hAPP(V717F)". The nucleotide sequence of the hAPP(V717F) mutant gene was confirmed by DNA sequencing.

<1-3> Preparation of βCTF99 mutant gene

The present inventors tried to prepare a protein, which includes V717F mutation in the 717th amino acid region of the human amyloid beta precursor protein represented by SEQ. ID. No 1 and contains the C-terminal amino acid sequence. Particularly, the C-terminal fragment (672nd - 751st) was amplified by PCR using cDNA (SEQ. ID. No 3) coding $APP751_{V717F}$ protein (SEQ. ID. No 4), which induces Indiana mutation in APP protein represented by SEQ. ID. No 2, as a template, resulting in a mutant gene. PCR was performed by using the vector containing hAPP(V717F) cDNA prepared in the above <Example 1-2> as a template with primer sets of app99f primer represented by SEQ. ID. No 24 (5'-cgaattcgatgcagaattcc-3') and appr-1r primer represented by SEQ. ID. No 7 with 32 cycles of

denaturation at 95℃ for 1 minute, primer annealing at 57℃ for 40 seconds and extension at 72℃ for 1 minute. The PCR product was digested with *EcoR* I and *Spe* I, which was linked to pBluscriptⅡ KS vector (Stratagene) pre-digested with *EcoR* I and *Spe* I. Thus, the C-terminal of APP began to carry mutation, and the mutant gene was represented by SEQ. ID. No 5 and coded a protein represented by SEQ. ID. No 10. The resulting mutant gene was then named "βCTF99". The nucleotide sequence of the mutant gene was confirmed by DNA sequencing.

In order to insert signal peptide in the above mutant gene, signal peptide region was amplified by PCR using pKS-aap696-1/2 vector bearing signal peptide as a template. The PCR was performed by using primer sets of app-sig-1f primer, represented by SEQ. ID. No 22, having *Bgl* II recognition site and app-sig-1r primer, represented by SEQ. ID. No 23, having *EcoR* I recognition site, with 32 cycles of denaturation at 95℃ for 1 minute, primer annealing at 55℃ for 1 minute and extension at 72℃ for 1 minute. The PCR product was digested with *Bgl* II, linearized by Klenow enzyme, digested with *EcoR* I, and then subcloned into *EcoR* I digesting region of pBluescript Ⅱ KS vector (Stratagene) digested with *BamH* I, linearized by Klenow enzyme and digested with *EcoR* I.

In order to enhance translation efficiency of the

signal peptide, PCR was performed by using pBluescript II KS vector harboring the signal peptide as a template with primer sets of app-koz-f primer represented by SEQ. ID. No 15 having *Xba* I recognition site and Kozac sequence (GACC) and app-koz-r primer represented by SEQ. ID. No 21 having *Not* I recognition site, followed by insertion of Kozac sequence (GACC) in front of starting codon (ATG) of the signal peptide. The PCR product was digested with *Xba* I and *Not* I, which was fused into pBluescript II KS digested with *Xba* I and *Not* I, resulting in the construction of pKS-kozappsig vector. The vector was digested with *EcoRI* and the constructed βCTF99 vector was digested with *EcoRI*. The both digested products were fused, leading to the preparation of a vector producing a fusion protein where signal peptide and βCTF99 were fused. So, the recombinant protein containing Kozac sequence, signal peptide and a gene coding βCTF99 protein in which Indiana mutation was induced was prepared and named "βCTF99(V717F)".

<Example 2> Construction of an expression cassette containing βCTF99(V717F) mutant gene for transgenic animal

In order to prepare an AD animal model, an expression cassette for transformation containing βCTF99(V717F) mutant gene was constructed. Particularly,

pGK-neo-PA vector (Lee *et al.*, *J. Neurosci.*, 2002, 15:7931-7940) was amplified using primer sets of SV40pA-f primer represented by SEQ. ID. No 13 (5'-tcccgcggtccagacatgataagatacattga-3', the underlined part presented *Sac* II restriction enzyme recognition site) and SV40pA-r primer represented by SEQ. ID. No 14 (5'-gttcgagctcataatcagccataccacatttg-3', the underlined part presented *Sac* I restriction enzyme recognition site), resulting in 247 bp sized SV40-pA fragment for polyadenylation signal of the mutant gene. Then, the fragnent was digested with *Sac* II and *Sac* I, which was inserted into pBluescript II KS vector. PsisCAT6a vector (Sasahara, M. *et al.*, *Cell*, 1991, 64(1):217-27) was digested with *Xba* I, linearized with Klenow enzyme, and digested with *Hind* III, resulting in human platelet-derived growth factor-beta (PDGF-beta) promoter fragment. The obtained PDGF-beta promoter fragment was inserted into pBluescript II KS vector that was digested with *Sal* I, linearized with Klenow enzyme and then digested again with *Hind* III. The pBluescript II KS vector bearing the above PDGF-beta promoter fragment was digested with *Kpn* I and *Hind* III, resulting in 1.5 kb sized PDGF-beta promoter fragment. And the fragment was inserted into *Kpn* I and *Hind* III recognition sites of pBlescript II KS vector containing SV40 pA region. The resulting vector, thus,

has a structure that has PDGF-beta promoter and SV40-pA region respectively at each side of multicloning site of pBluescript II KS vector. In the vector, βCTF99(V717F) mutant gene containing Kozac sequence (GACC) in front of starting codon, prepared in the above <Example 1>, was inserted, resulting in an expression cassette for transformation. Finally, the expression cassette was constructed to possess PDGF-β promoter-βCTF99(V717F)-pA in that order, and named "PDGF-βCTF99(V717F)-pA" (Fig. 1A).

<Example 3> Construction of an expression cassette containing intron and βCTF99(V717F) mutant gene for transgenic animal

The intron elevates expression efficiency of a mutant gene and increases transcription stability. Thus, in order to introduce a mutant gene into an animal model effectively, the present inventors introduced the intron B gene (918 bp)(Choi *et al.*, *Molecular and cellular biology*, June 1991, p.3070-3074; Palmiter *et al.*, *PNAS*, 1991, 88:478-482) of human β-globin gene into the expression cassette prepared in the above <Example 2>. Precisely, the intron B of human β globin gene was amplified by PCR using the primers of hglob-f represented by SEQ. ID. No 16 and hglob-r represented by SEQ. ID. No 17 and genomic DNA,

which was obtained from the human neuroblastoma cell line SH-SY5Y, as a template. The amplified intron B gene product derived from human β-globin gene was sub-cloned into pGEM-T Easy vector (Promega, Madison, WI, USA), which was inserted between PDGF-β promoter gene of PDGF-βCTF99(V717F)-pA expression cassette constructed in the above <Example 2> and βCTF99(V717F) mutant gene. The resulting expression vector for transformation was named "PDGF-βCTF99(V717F)-pA" (Fig. 1A).

<Example 4> Generation of transgenic animals

PDGF-βCTF99(V717F)-pA expression cassette constructed in the above <Example 2> and PDGF-intron-βCTF99(V717F)-pA expression cassette constructed in the above <Example 3> were digested with a restriction enzyme (*BssH*II), resulting in 3.1 kb sized linearized fragment. The product was microinjected into the pronuclei of fertilized eggs prepared from inbred C57BL/6 mice. After the microinjection, the fertilized eggs were transferred to the oviduct of pseudopregnant female (ICR) mice. The methods for transformation of animals used in the present invention including microinjection were in accordance with the conventional methods (Games *et al.*, *Nature*, 1995; Hisao *et al.*, *Science*, 1996).

<Example 5> Confirmation of the insertion of a mutant gene into chromosomal DNA

Genomic DNA was extracted from the tails of F1 mice generated from the animal transformation procedure performed in the above <Example 4>, and PCR was performed to confirm whether or not a mutant gene was rightly inserted into nuclei of fertilized eggs. Particularly, PCR was performed with primer sets of trapp-fs primer represented by SEQ. ID. No 18 and trapp-r1 primer represented by SEQ. ID. No 19, in order to investigate the insertion of a mutant gene into the F1 mice generated by using PDGF-βCTF99(V717F)-pA expression cassette excluding intron. In the meantime, another PCR was performed with primer sets of trint-f1 primer represented by SEQ. ID. No 20 and sv40pA-r primer represented by SEQ. ID. No 14, in order to investigate the insertion of the mutant gene in the F1 mice generated by using an expression cassette including intron (PDGF-intron-βCTF99(V717F)-pA).

As a result, among the F1 mice generated by the introduction of an expression cassette excluding intron (PDGF-βCTF99(V717F)-pA), 16 mice were confirmed to bear the expression cassette. In the case of F1 mice generated by the introduction of an expression cassette including intron (PDGF-intron-βCTF99(V717F)-pA), only 2 mice were

confirmed to bear the expression cassette.

Southern blot analysis was also performed to confirm the introduction of the expression cassette of the present invention. Precisely, genomic DNA was extracted from the tails of F1 mice generated from the animal transformation procedure taken in the above <Example 4>, and then 15 μg of the genomic DNA was digested with restriction enzyme *Spe* I. The resulting products were electrophorezed on agarose gel, and then transferred onto nitrocellulose membrane. Hybridization was performed using a ^{32}P-labeled probe prepared from the 350 bp *SpeI* fragment at the C-terminus of APP cDNA, and the results were developed on X-ray film.

The results resembled those of the above PCR with the genomic DNA, that is, among the F1 mice generated by the introduction of an expression cassette excluding intron (PDGF-βCTF99(V717F)-pA), 16 mice were confirmed to bear the expression cassette. In the case of those F1 mice generated by the introduction of an expression cassette including intron (PDGF-intron-βCTF99(V717F)-pA), only 2 mice were confirmed to bear the expression cassette (Fig. 1B).

The transgenic mice which were confirmed by genomic

PCR and Southern blotting, to bear the βCTF(V717F) mutant gene of the present invention were inbred with C57BL/6 mice.

<Example 6> Investigation of the transgene expression

In order to confirm whether or not βCTF99(V717F) mutant gene was successfully introduced and expressed in the transgenic mice of the present invention, total RNA was prepared from the brains of transgenic mice, followed by Northern blotting. Northern blot analysis was performed according to the method of Lee, *et al.* (Lee *et al.*, *J Neurosci*, 2002, 15:7931-7940). Precisely, total RNA was prepared from the brains of wild type and transgenic mice at 2 months, which were confirmed to have βCTF99(V717F) mutant gene transducted in the above <Example 4> and <Example 5>. Trizol reagent (Sigma, St. Louis, MO, USA) was used for the extraction of the total RNA. A membrane blot carrying 30 μg of total RNA was prepared after separating on denaturing agarose gel (1% agarose, 6.2% formaldehyde in 1× MOPS), and hybridized with a ^{32}P-labeled probe prepared from the *Spe*I-digested fragment (350 bp) of CTF99, which was also used in the above <Example 5> for Southern blot analysis, and then the results were developed on X-ray film. The probe was able to recognize both internal APP transcript and

βCTF99(V717F) mutant transcript.

As a result, the expression of APP mutant transcript was much higher as a whole than that of the endogenous APP transcript in transgenic mice, regardless of that βCTF99(V717F) mutant gene transforming a mouse included intron or not. In particular, the expression of βCTF99(V717F) was especially higher in transgenic mice (F17 mice) bearing βCTF99(V717F) mutant gene harboring intron (Fig. 1C). And those transgenic mice showing the high expression of βCTF99(V717F) mutant gene including intron were named "Tg-βCTF99/B6", which were, from then on, used for further experiments of the present invention.

The transgenic mouse Tg-βCTF99/B6 was deposited at Korean Collection for Type Cultures (KCTC) of Korea Research Institute of Bioscience and Biotechnology (KRIBB) on March 10, 2003 (Accession No: KCTC 10609BP).

<Example 7> Protein production by the transgene

It was confirmed in the above <Example 6> that Tg-β CTF99/B6 mice of the present invention expressed β CTF99(V717F) mutant gene successfully. In order to investigate the possibility of protein production by the expressed gene, total protein was extracted from the brains of wild type controls and Tg-βCTF99/B6 mice at 4 -

5 months, followed by Western blotting. The Western blot analysis was performed according to the method of Lee, *et al.* (Lee *et al.*, *Brain Res Mol Brain Res*, 1999, 70:116-124). Particularly, mouse brain tissue was homogenized in 4℃ lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate) containing 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor (Complete™; Roche, Mannheim, Germany). Centrifugation was performed with the homogenized brain tissue samples at 13,000 rpm, for 20 minutes at 4℃ to obtain supernatant. The protein in the supernatant was quantified by BCA quantification kit (Sigma, St. Louis, MO, USA). Each lane was loaded with 30 ㎍ of the protein, and acrylamide gel electrophoresis was carried out. The separated proteins were transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA) and the membranes were blocked with 5% non-fat dry milk, 2% BSA, 4% FBS, 4% horse serum, 4% goat serum in Tris-buffered saline and 0.1% Tween 20. Two βCTF-specific polyclonal antibodies commonly detected the endogenous ~12 kD βCTF99 (A8717; Sigma, St. Louis MO, USA) and ~10 kD αCTF83(p3) (51-2700; Zymed, San Francisco, CA, USA) fragments were used for the Western blot analysis to confirm the production of the βCTF99(V717F) mutant protein. The βCTF99 and αCTF83 are proteins generated by β-secretase and α-secretase, respectively, in the brains

of non-transgenic controls. Immunoblots were detected using ECL detecting reagents (Santa Cruz, CA, USA).

For the detection of βCTF99, the brain tissues were homogenized in 1:10 (g/vol) Tris-buffered saline (TBS) containing 50 mM Tris-HCl (pH 8.0), 175 mM NaCl, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Complete™; Roche, Mannheim, Germany). Fifty μg of the protein sample were mixed with an equal volume of 2× Laemmli sample buffer containing 10% β-mercaptoethanol, boiled for 10 min, and then electrophoresed on 16.5% Tris/tricine agarose gel as described (Li *et al.*, 1999). After being transferred onto PVDF membranes, the resolved proteins were probed with polyclonal anti-CTF. Immunoblots were detected using ECL detection reagents.

As a result, in the Tg-βCTF99/B6 brain at 4-5 months, the amounts of βCTF99 and αCTF83 were notably up-regulated. Densitometric measurements of the CTFs using computer-assisted imaging software indicated that the expression levels of βCTF99 and αCTF83 were 2.63±0.37 and 2.61±0.2 fold that of endogenous βCTF99 and αCTF83 (Fig. 2A).

<Example 8> Immunohistochemical analysis of the brains of transgenic mice

For immunohistochemical experiments, the mice were perfused with 0.9% saline through ascending aorta, and then perfused again with 4% paraformaldehyde in 0.1 M phosphate buffer (referred "PB" hereinafter, pH 7.4). The brain was removed and fixed in the fixative at 4℃. The fixed brain was coronally cut into 40 ㎛-thick sections with a vibratome. The sections were reacted in 3% hydrogen peroxide solution dissolved in 0.1 M PB (pH 7.4) for 30 minutes and washed with PB. The sections were blocked by 5% normal goat serum, 2% BSA and 2% FBS for 2 hours at room temperature. The primary antibody was added to the blocking buffer, which was left at 4℃ for overnight for reaction. After washing with PB solution, the secondary antibody, which was biotinylated by being diluted 1:200 fold, was added. Then, 1:100 fold diluted avidin and biotinylated HRP complex (Vector Laboratories, Burlingame, CA) were also added for one more hour reaction. 0.05% 3,3'-diaminobenzidine and 0.001% hydrogen peroxide in 0.1 M Tris (pH 7.4) were used for the color development. Cerebral cortex (referred "CX" hereinafter), pyramidal cells of CA1-CA3 regions (referred "CA1"-"CA3" hereinafter), hippocampus (referred "HP" hereinafter) and dentate gyrus (referred "DG" hereinafter) were used for

the analysis.

As a result, increased expression of βCTF99 protein was observed in neuronal cells of broad brain regions including cerebral cortex in Tg-βCTF/B6 mice of the present invention (Fig. 2B-C). However, plaque like-Aβ deposition was not found in the brains of Tg-βCTF/B6 mice at upto 18 months. And, approximately 92% of the Tg-βCTF/B6 mice survived until at least 480 days, suggesting that those transgenic mice can survive longer than the conventional transgenic mice, eventhough the lethality is elevated compared to that of the wild type control mice. Thus, the transgenic mice of the present invention are much effective as animal models.

<Example 9> Expressions of other proteins in the brains of transgenic mice

Expressions of other proteins that might be affected by βCTF99(V717F) mutant gene introduced into Tg-βCTF99/B6 mice of the present invention were investigated. Particularly, Western blot analysis was performed with brain tissues by the same way as described earlier in the <Example 7>. The antibodies used for the analysis were anti-phospho-JNK antibody (9251S; Cell Signaling, Beverly, MA, USA), anti-phospho-c-Jun antibody (9261S; Cell

Signaling), anti-phospho-p38 antibody (9211S; Cell Signaling), anti-JNK3 antibody (06-749; Upstate Biotechnology, Lake placid, NY, USA), anti-CREB antibody (Upstate Biotechnology), anti-phospho-CREB antibody (Upstate Biotechnology), anti-MAP2 antibody (Upstate Biotechnology), anti-calbindin antibody (C9848; Sigma, St. Louis, MO, USA), anti-parvalbumin (P3088; Sigma), anti-calretinin antibody (AB5054; Chemi-Con, Temecula, CA, USA), anti-JNK1 antibody (15701A; Pharmingen, San Diego, CA, USA), anti-JNK2 antibody (sc-572; Santa Cruz Bio-Technology, Santa Cruz, CA, USA), anti-phospho-ERK antibody (sc-7383; Santa Cruz Bio-Technology), anti-ERK antibody (sc-154; Santa Cruz Bio-Technology), anti-Bcl-2 antibody (sc-783; Santa Cruz Bio-Technology), anti-Bad antibody (sc-942-G), anti-Bax antibody (sc-6236; Santa Cruz Bio-Technology), and anti-Bcl-xL (sc-7195; Santa Cruz Bio-Technology).

Recent reports indicate that the human AD brain shows phospho-JNK up-regulation (Zhu *et al.*, 2001, *J Neurochem.*, 76:435-441; Savage *et al.*, 2002, *J Neurosci.*, 22: 3376-3385). Thus, the present inventors performed Western blot analysis to examine the expression of phospho-JNK protein in the brain of the transgenic mouse of the present invention. As a result, the expressions of

phospho-JNK protein and phospho-c-Jun protein in the brain of Tg-βCTF99/B6 at 15 months were higher than those of age-matched wild type controls, whereas the expressions of JNK1, JNK2, JNK3, phospho-ERK and phospho-p38 were not significantly changed (Fig. 3).

Bcl-2 and Bcl-xL proteins are anti-apoptotic whereas Bax and Bad proteins are pro-apoptotic. And these are B-cell leukemia-2 (Bcl-2) family proteins (Davies *et al.*, 1995, *Trend Neurosci.*, 18:355-358). The present inventors also performed Western blot analysis to detect the change of expression level of Bcl-2 family protein in the brain of the transgenic mice. As a result, the expressions of Bcl-2, Bad and Bax were significantly elevated, whereas Bcl-2-xL expression was attenuated in Tg-βCTF99/B6 brain at 14-16 months (Fig. 4A). The result indicates that the expression of Bcl protein is affected by the insertion of βCTF99(Ld) mutant gene of the present invention. Based on the result, immunohistochemical analysis was performed to examine the expressions of Bad and Bax in CA1 region, the pyramidal cell layer of the hippocampus, in analogy to the procedure as described in the <Example 8>. Consistent with the results of Western blot analysis above, the expressions of Bad and Bax proteins in CA1 region were increased (Fig. 4B).

It has been known that AD brain shows the increased

expression of calcium-binding proteins (Anthony *et al.*, 1990, Proc. Natl. Acad. Sci USA., 87:4078-4082; Mikkonen *et al.*, 1999, *Neuroscience*, 92:515-532; Bu *et al.*, 2003, *Exp Neurol.*, 182:220-231). Thus, the expressions of calcium-binding proteins such as calbindin, parvalbumin and calretinin were investigated by Western blot and immunohostochemical assay. As a result, calbindin expression was reduced in hippocampus, CA1, CA3 and DG regions of Tg-βCTF99/B6 at 14-16 months, compared to that of the wild type controls (Fig. 5A-Western blot, Fig. 5B-Immunohistochemical assay). Calbindin expression was not detected in the brains of Tg-βCTF99/B6 at 4-5 months. In the meantime, parvalbumin and calretinin expressions were not much different from those of wild type controls.

Recent reports indicate that the phospho-CREB level is reduced in the AD brain, which is nothing to do with the variations of total CREB protein level, though (Yamamoto-Sasaki *et al.*, 1999, *J Neurosci.*, 22:1858-1867). Especially, the increased expression of CREB protein during neuronal activity induces synaptic plasticity, in particular hippocampus-based memory retention (Mayford *et al.*, 1999, *Trends Genet.*, 15:463-470; Colombo *et al.*, 2003, *J Neurosci.*, 23:3547-3554; Viola *et al.*, 2000, *J Neurosci.*, 20: RC112 (1-5)). Accordingly, the present inventors performed Western blot and immunohistochemical analysis to

examine phospho-CREB protein expression at transgenic mice of the present invention. As a result, total CREB protein expression was not changed in the brain of Tg-βCTF99/B6 at 14-16 months, whereas phospho-CREB protein expression was reduced in hippocampus, CA1 and CX regions of the transgenic mice, compared to that of the wild type controls (Fig. 6A-Western blot, Fig. 6B-K-Immunohistochemical analysis). However, phospho-CREB protein expression in the brain of Tg-βCTF99/B6 at 5-7 months was similar to that of wild type controls.

In order to examine the possibility of neuronal loss by βCTF99(V717F) mutant gene of the present invention, the expression of neuron-specific marker MAP-2 protein was measured. As a result, the expression of MAP-2 protein was reduced in CX and hippocampus CA1 region of the brain of Tg-βCTF99/B6 at 15-18 months, indicating that the mutant gene had influence on neuronal formation (Fig. 7A-H). However, the level of the protein in the brain of the transgenic mouse at 7 months was not much different from that of a wild type control.

In order to examine the possibility of neuronal degeneration by βCTF99(Ld) mutant gene of the present invention, the expression of Neu protein was investigated. As a result, neuronal cell density was approximately 5-10% reduced at the transgenic mice at 11-12 months, which went

further to 25% reduction at 18 months. The result indicates that βCTF99(Ld) mutant gene of the present invention induces gradual neuronal degeneration(Fig. 7I).

<Example 10> Cognitive function of the transgenic mice

Histopathological characteristics of AD brain are (1) the deposition of extracellular senile plaques, (2) the formation of intracellular neurofibrillary tangle, (3) the degeneration of axons and synapses, and neuronal loss, and (4) malfunction of the brain by neuronal loss, which are all detectable by histological test. In particular, cognitive deficits are the most characteristic and important morphological and clinical symptom. Thus, it is important for an AD animal model to show not only histological characteristics including senile plaques deposition, but also, in fact more importantly, cognitive deficits. The present inventors performed Morris water maze test, passive avoidance test, and open field test to judge the cognitive deficits in candidates for AD models.

Mice were housed in cages in a temperature- and humidity-controlled environment with a 12 hour-light/dark cycle (light switched on at 7 a.m.). All animals were handled in accordance with the animal care guideline of Ewha Womans University School of Medicine. To track the animals' behavior, a computerized video-tracking system

(SMART; Panlab S. I., Barcelona, Spain) was used.

Two-sample comparisons were carried out using the Student t-test, while multiple comparisons were made using one-way ANOVA followed by the Newman-Keuls multiple range test. All data were presented as the means±S.E.M. The statistical differences were accepted at the 5% level unless otherwise indicated.

<10-1> Open field test

Locomotor activity was measured in the open field of a white Plexiglas chamber (45×45×45 cm). Illumination in the chamber was adjusted to 70 lux. The mice were all placed in the same environment as that of the chamber 30 minutes prior to the test. Each mouse was placed individually in the middle of the open field and locomotion was recorded for 60 minutes. The horizontal locomotor activity was judged according to the distance the animal moved. The inner 30 percentage of the open filed was defined as the center of the chamber.

As a result, the locomotor activities shown by Tg-βCTF99/B6 at 7-11 months were similar to those of wild type controls. The locomotor activity displayed by Tg-βCTF99/B6 at 14 month was slightly elevated, compared to that of the wild type controls, though it was not

significant (Fig. 8A). The approaches to the center of the open field (a sign of anxiety) were also similar to those of control mice.

<10-2> Rota-rod test

Rota-rod test was performed to evaluate motor coordination and motor learning. Rota-rod consists of a rotating cylinder (4.5 cm in diameter) with a speed controller attached. Mice were placed on the top of the cylinder where they have access to tight grip. Rota-rod was spinned at the speed of 5-20 rpm, and the speed was gradually increased. Cut-off time was set as 3 minutes and intertrial interval was 60 minutes. Hang-on time on rod was measured.

As a result, motor coordination of the transgenic mice of the present invention at 5.5 months was similar to that of wild type controls. However, motor coordination of the transgenic mice of the present invention at 11 months was reduced, compared to that of the wild type controls, though the difference was not significant (Fig. 8B).

<10-3> Morris water maze test

Morris water maze test is a hippocampus-dependent

analysis method that depends largely on the capability of an animal to learn and remember the relation between stimulus at a distance and hidden platform for escape (Morris *et al.*, 1982, *Nature*, 297, 701). That is, in this test, forced swimming or the latency to find a hidden platform by taking advantage of spatial indices memorized during placing on the platform was observed. Based on this observation, cognitive function of a mouse was investigated and quantified by comparison of the distance and the time that the mouse swam. In order to investigate memory retention of a mouse, the locations of the entrance to the pool and the hidden platform were changed often, while spatial indices were still located same. Particularly, water maze consisted of a 90 cm-diameter cylinder pool filled with 22℃ opaque milky water. A 10 cm-diameter hidden platform was placed in a quadrant 1.5 cm below the surface of the opaque water. The pool was placed in a room with abundant environmental and artificial cues including a window, a chair and posters. In the course of daily testing, mice were admitted successively into each of the quadrants and allowed to swim for 90 seconds maximum. On locating the platform, the animals were permitted to remain on it for 30 seconds before the session was terminated. The latency to find the platform for each of two trails and the average of the

two trails were recorded for each mouse.

As a result, wild type controls at 7, 11 and 14 months could recognize the indices of the hidden platform, and this achievement improved trial after trail. On the other hand, Tg-βCTF99/B6 mice at 7, 11 and 14 months showed longer latency to find the hidden platform, compared to the wild type controls, indicating the cognitive deficits, eventhough the difference was not very significant (Fig. 9A-B). In the meantime, swimming speeds of Tg-βCTF99/B6 mice at 7, 11 and 14 months were similar to those of age-matched controls (Fig. 9C). Those results indicate that Tg-βCTF99/B6 mice show elevated cognitive deficits, compared to the wild type controls.

<10-4> Passive avoidance test

Mice prefer darkness to lightness. When mice are allowed to choose one of the two chambers, one is lighted and the other is dark chamber, they have no hesitation to go for the dark chamber. Mice are once placed in a lighted chamber and then allowed to move to a dark chamber but a strong electric shock is given then (that is, a training). After the training, when mice are forced to select a chamber to enter, most of wild type mice try to stay in a lighted chamber without the electric shock,

eventhough unwillingly. Passive avoidance test is designed based on the above idea, and so the test is to investigate learning and memory retention through spatial information such as a lighted and a dark chamber, and an electric shock.

Particularly, the test apparatus of the invention consisted of a brightly lit and a dark compartment (15 x 15 x 15 cm each), each equipped with a shock-grid floor, and a door between the two chambers. During the first day of testing, each mouse was placed in the lighted chamber and left to habituate to the apparatus for 5 minutes, while allowing it to explore the light and dark rooms. On the second day, the mice were placed in the lighted chamber. After 30 seconds, the middle door was opened and the latency for the mouse to enter the dark chamber was measured. When the mouse entered the dark room, the door was closed and two successive electric foot-shocks (100 V, 0.3 mA, 2 seconds) were delivered through the grid-floor. After training, mice were individually replaced in the lighted chamber and the latency to enter the dark chamber was measured.

As a result, pre-shock latency to enter the dark chamber of wild type control mice at 7 - 14 months was similar to that of Tg-βCTF99/B6 mice of the present

invention. However, the post-shock latency to enter the dark chamber of Tg-βCTF99/B6 mice of the present invention was much shorter than that of wild type control mice (Fig. 9D). The results indicate that the transgenic mice of the present invention show cognitive deficits.

<10-5> Elevated plus maze test

Increased anxiety is a problematic symptom of human AD patients (Folstein and Bylsma, 1999, Alzheimer Disease (Eds by Terry et al.,) 2nd. Lippincott Williams & Wilkins, Philadelphia). So, the present inventors needed to investigate the possibility of increased anxiety by the introduction of APP mutant gene in Tg-APP/B6 transgenic mice. Elevated plus maze apparatus consisted of four arms (30 x 7 cm) made of black Plexiglas, which were placed at right angles to each other and elevated 50 cm above the floor. Two of the arms had 20 cm high walls (enclosed arms), while other two had no walls (open arms). The illumination at the center was adjusted to 40 lux. For the test, the mouse was initially placed at the center of the platform and left to explore the arms for 5 minutes. The number of entries in the open and in the enclosed arms and the time spent in each arm was recorded. Entry into each arm was scored as an event if the animal placed all four paws into the corresponding arm.

As a result, the number of entries into open and enclosed arms for Tg-βCTF99/B6 at 7 months was similar to that of age-matched controls. However, the number of entries and the time spent in the open arm for the Tg-βCTF99/B6 transgenic mice at 13 months was less than that of age-matched controls. The results indicate that Tg-βCTF99/B6 mice of the present invention show increased anxiety (Fig. 10).

[Industrial Applicability]

As explained hereinbefore, unlike the wild type control mice, the transgenic mice of the present invention showed notable cognitive deficits, meaning the impaired memory retention, in Morris water maze test. In addition, in elevated plus maze test, the transgenic mice of the present invention showed increased anxiety. Those results confirmed that the transgenic mice of the present invention showed clinical symptoms of AD better than any other conventional AD animal models. The transgenic mice of the present invention showed age-dependent neuronal loss, which is superior to any known conventional AD animal models. Therefore, the transgenic mice of the present invention are expected to serve as a useful AD

model for the study of AD-related pathogenesis including the study of cognitive deficits.

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : HAN, Byung Lim
Institute of Neuroscience, Ewha Womans University Medical School,
#70, Jungmo 6-gil, Jungnang-gu, Seoul 110 783,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:

-Tg-CTF99/H6 (mouse embryo)

Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:

KCTC 10609BP

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:
[x] a scientific description
[ ] a proposed taxonomic designation
(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **March 10 2004.**

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: **Korean Collection for Type Cultures**

Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB)
#52, Oun-dong, Yusong-ku,
Taejon 305-333,
Republic of Korea

Signature(s) of person(s) having the power to represent the International Depositary Authority or authorized official(s):

PARK, Yong-Ha Director
Date: **March 31 2004**

Form BP/9 (KCTC Form 17) side one

【CLAIMS】

【Claim 1】

A vector, for transformation of animals to induce Alzheimer's disease pathology, that contains a gene coding a protein represented by SEQ. ID. No 10 containing C-terminal fragment (CTF) of mutant human amyloid beta precursor protein (APP) in which 698th amino valine (V) of AP751 is replaced with phenylalanine (F).

【Claim 2】

The vector for transformation of animals to induce Alzheimer's disease pathology as set forth in claim 1, wherein the vector additionally includes a promoter and polyadenylation region.

【Claim 3】

The vector design for transformation of animals to induce Alzheimer's disease pathology as set forth in claim 2, wherein the promoter is human PDGF-β promoter.

【Claim 4】

The vector for transformation of animals to induce Alzheimer's disease pathology as set forth in claim 2, wherein the polyadenylation region is SV40 pA.

【Claim 5】

The vector for transformation of animals to induce Alzheimer's disease pathology as set forth in claim 2, wherein the vector additionally includes Kozac sequence between a promoter and a gene coding C-terminal fragment of the mutant human amyloid beta precursor protein.

【Claim 6】

The vector for transformation of animals to induce Alzheimer's disease pathology as set forth in claim 2, wherein the vector additionally includes nucleotide sequence coding signal peptide in front of a gene coding C-terminal fragment of mutant human amyloid beta precursor protein.

【Claim 7】

The vector for transformation of animals to induce Alzheimer's disease pathology as set forth in claim 6, wherein the nucleotide sequence is represented by SEQ. ID. No 25.

【Claim 8】

The vector for transformation of animals to induce Alzheimer's disease pathology as set forth in claim 2, wherein the vector is designed to include human PDGF-β

promoter gene, mutant gene coding an amino acid sequence represented by SEQ. ID. No 3 and SV40 pA in that order, and represented by the cleavage map PDGF-βCTF99(V717F)-pA.

【Claim 9】

The vector for transformation of animals to induce Alzheimer's disease pathology as set forth in any of claim 2 ~ claim 7, wherein the vector additionally includes intron between a promoter gene and a mutant gene coding a mutant protein.

【Claim 10】

The vector for transformation of animals to induce Alzheimer's disease pathology as set forth in claim 9, wherein the intron is intron B that is derived from human beta-globin gene.

【Claim 11】

The vector for transformation of animals to induce Alzheimer's disease pathology as set forth in claim 9, wherein the vector is designed to include human PDGF-β promoter gene, intron B gene of human beta-globin, mutant gene coding an amino acid sequence represented by SEQ. ID. No 3 and SV40 pA in that order, and represented by the cleavage map PDGF-intron-βCTF99(V717F)-pA.

【Claim 12】

A transgenic mouse with induced Alzheimer's disease pathology generated by introducing the vector for transformation of animals of claim 1.

【Claim 13】

The transgenic mouse with induced Alzheimer's disease pathology as set forth in claim 12, wherein the mouse is Tg-βCTF/B6 showed clinical symptoms of AD such as motor coordination deficit, impaired memory retention, cognitive deficits and increased anxiety (Accession No: KCTC 10609BP).

FIGURES

FIG. 1

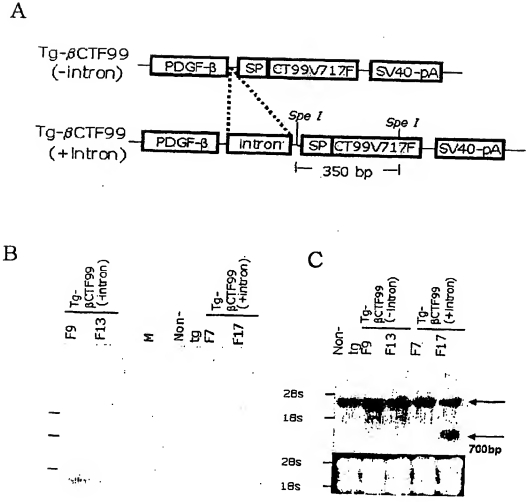
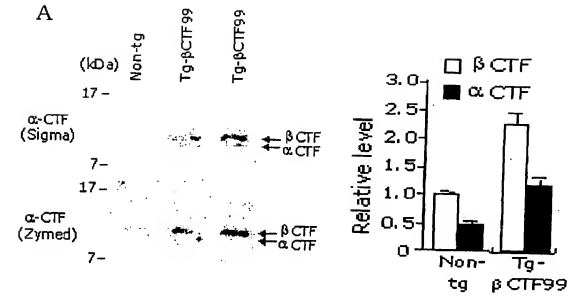


FIG. 2


A
(kDa)
Non-tg
Tg-βCTF99
Tg-βCTF99
17-
α-CTF
(Sigma)
7-
17-
α-CTF
(Zymed)
7-
β CTF
α CTF
β CTF
α CTF
β CTF
α CTF
Relative level
3.0
2.5
2.0
1.5
1.0
0.5
0
Non-tg
Tg-βCTF99

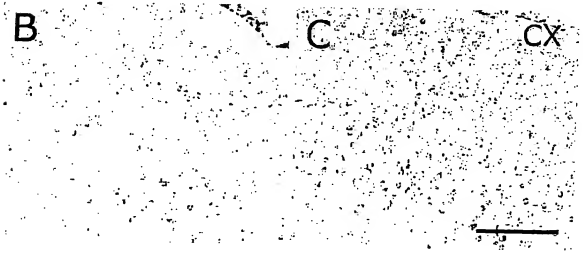

B
C
CX

FIG. 3

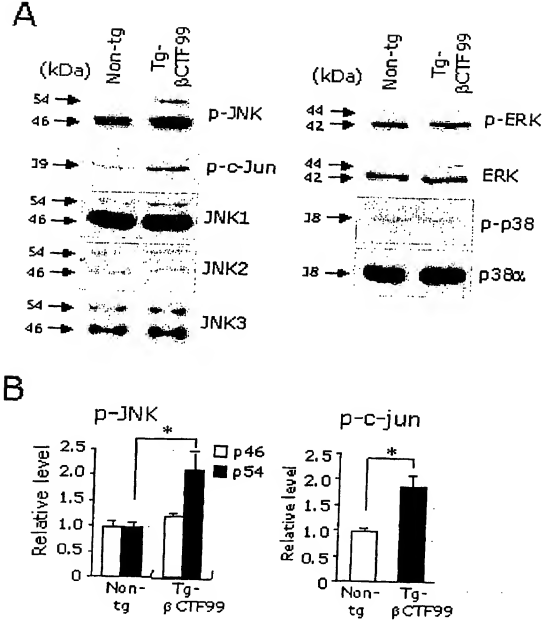

A
(kDa)
Non-tg
Tg-βCTF99
54
46
p-JNK
39
p-c-Jun
JNK1
JNK2
JNK3
44
42
p-ERK
ERK
38
p-p38
p38α
B
p-JNK
p-c-jun
Relative level
2.5
2.0
1.5
1.0
0.5
0
p46
p54
*
Non-tg
Tg-βCTF99

FIG. 4

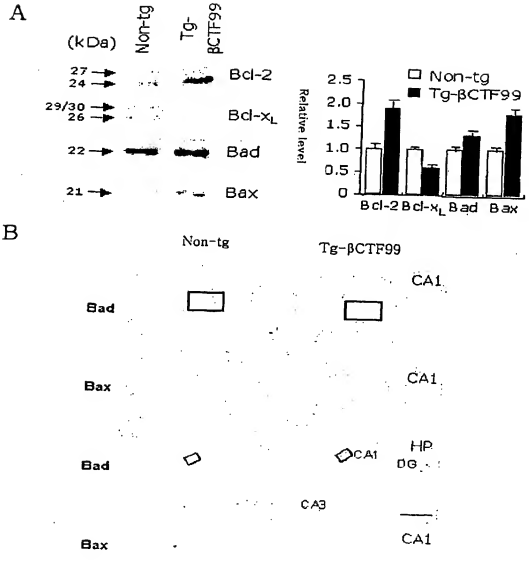
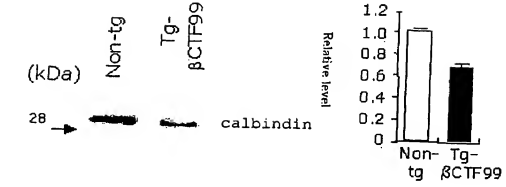

A
(kDa)
Non-tg
Tg-βCTF99
27
24
Bcl-2
29/30
26
Bcl-xL
22
Bad
21
Bax
Non-tg
Tg-βCTF99
Relative level
2.5
2.0
1.5
1.0
0.5
0
Bcl-2 Bcl-xL Bad Bax
B
Non-tg
Tg-βCTF99
Bad
CA1
Bax
CA1
Bad
CA1
HP
DG
CA3
Bax
CA1

FIG. 5

A


(kDa)
Non-tg
Tg-βCTF99
28
calbindin
Relative level
1.2
1.0
0.8
0.6
0.4
0.2
0
Non-tg
Tg-βCTF99

B

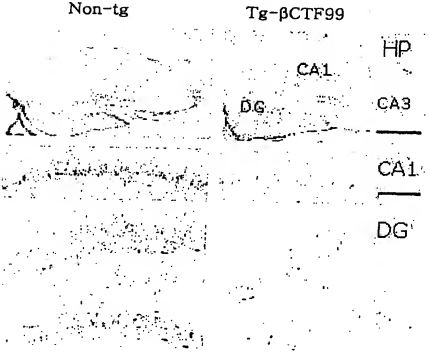

Non-tg
Tg-βCTF99
HP
CA1
DG
CA3
CA1
DG

FIG. 6

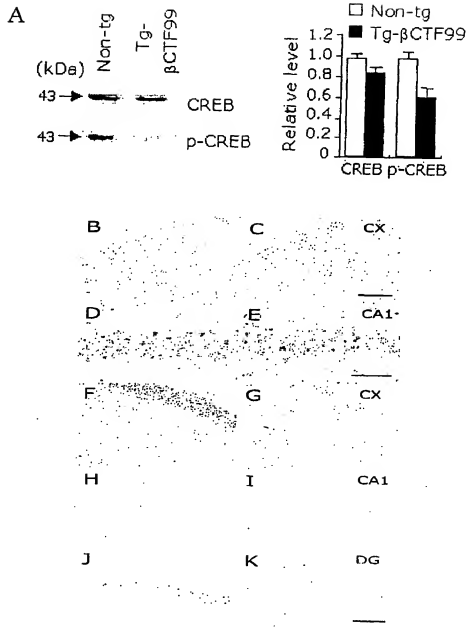
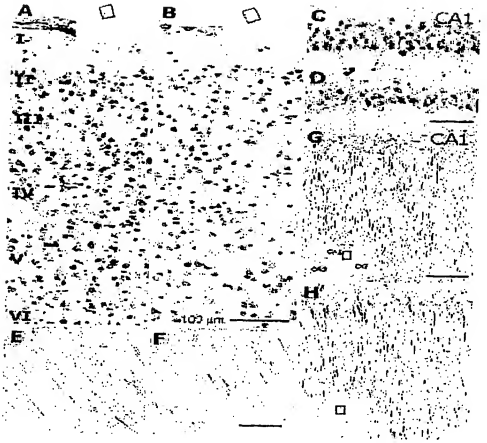

A
Non-tg
Tg-βCTF99
(kDa)
43
CREB
43
p-CREB
Non-tg
Tg-βCTF99
Relative level
1.2
1.0
0.8
0.6
0.4
0.2
0
CREB p-CREB
B
C
CX
D
E
CA1
F
G
CX
H
I
CA1
J
K
DG

FIG. 7


A
B
C
CA1
I
II
III
D
G
CA1
IV
V
H'
VI
100 μm
E
F

I

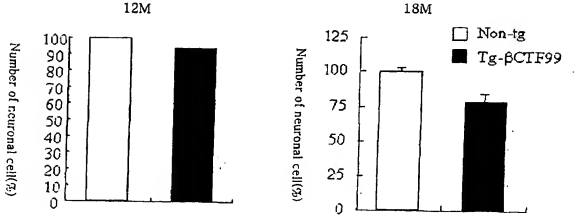

12M
18M
Number of neuronal cell(%)
100
90
80
70
60
50
40
30
20
10
0
125
100
75
50
25
0
Non-tg
Tg-βCTF99

FIG. 8

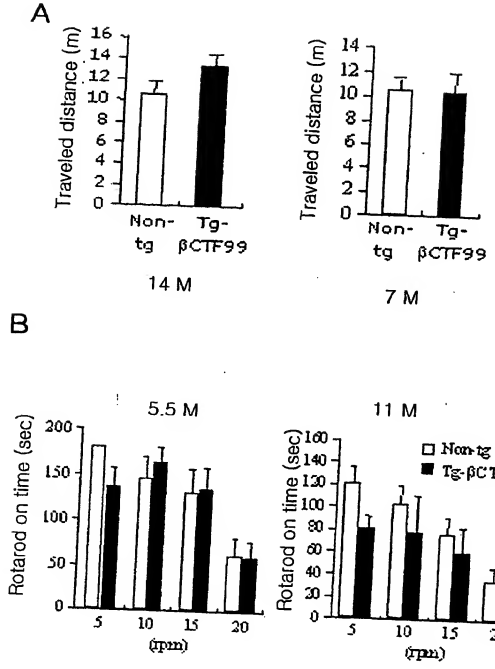

A
Traveled distance (m)
16
14
12
10
8
6
4
2
0
Non-tg
Tg-βCTF99
14 M
Traveled distance (m)
14
12
10
8
6
4
2
0
Non-tg
Tg-βCTF99
7 M
B
5.5 M
Rotarod on time (sec)
200
150
100
50
0
5
10
15
20
(rpm)
11 M
Rotarod on time (sec)
160
140
120
100
80
60
40
20
0
Non-tg
Tg-βCTF99
5
10
15
20
(rpm)

FIG. 9

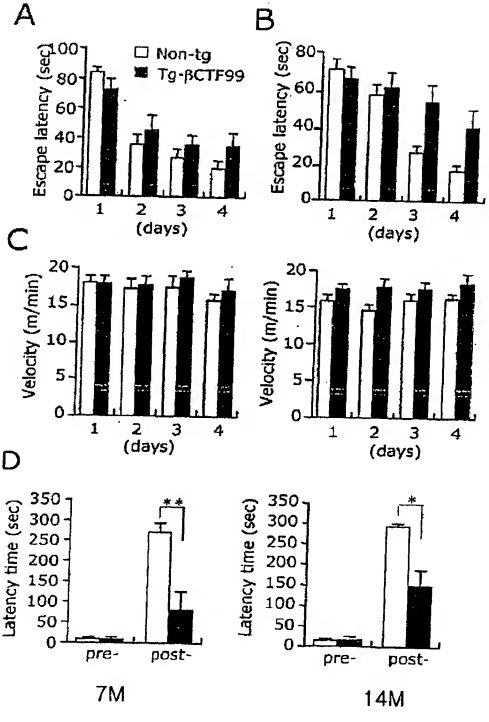
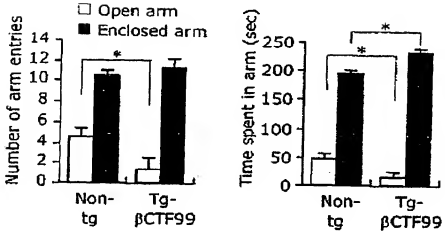

A
B
Non-tg
Tg-βCTF99
Escape latency (sec)
(days)
C
Velocity (m/min)
D
Latency time (sec)
pre-
post-
7M
14M

FIG. 10


Open arm
Enclosed arm
Number of arm entries
14
12
10
8
6
4
2
0
*
Non-tg
Tg-βCTF99
Time spent in arm (sec)
250
200
150
100
50
0
*
*
Non-tg
Tg-βCTF99

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2005/000969

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7 C12N 15/63, A01K 67/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean patents and applications for inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKIPASS, Delphion, NCBI PubMed, GenBank database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	R. GERLAI, ET. AL. "Behavioral Impairment of APP(V717F) mice in fear conditioning: is it only cognition?." In: Behav. Brain Res., (November 2002) Vol.136(2):503-509, see the whole document.	1-13
Y	E. M. ROCKENSTEIN, ET. AL. "Levels and Alternative Splicing of Amyloid β Protein Precursor (APP) Transcripts in Brains of APP Transgenic Mice and Humans with Alzheimer's Disease." In: J. Biol. Chem., (24 November 1995) Vol.270(47):28257-28267, see the abstract, figure 1, 2, and Materials and Methods.	1-13
Y	B. P. F. RUTTEN, ET. AL. "No alterations of hippocampal neuronal number and synaptic bouton number in a transgenic mouse model expressing the β -cleaved C-terminal APP fragment." In: Neurobiol. Dis., (March 2003) Vol.12(2):110-120, see the abstract and Materials and Methods.	1-13
Y	T. KAWARABAYASHI, ET. AL. "Accumulation of β -Amyloid Fibrils in Pancreas of Transgenic Mice." In: Neurobiol. Aging, (March-April 1996) Vol.17(2):215-222, see the abstract, figure 1, and Methods.	1-13
A	US 6717031 B2 (KATE DORA GAMES; DALE BERNARD SCHENK; LISA CLAIRE MCCONLOGUE; PETER ANDREW SEUBERT; RUSSELL E. RYDEL) 6 April 2004 (2004-04-06), see the whole document.	1-13

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 JUNE 2005 (27.06.2005)	**27 JUNE 2005 (27.06.2005)**
Name and mailing address of the ISA/KR Korean Intellectual Property Office 920 Dunsan-dong, Seo-gu, Daejeon 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Authorized officer AHN, Kyu Jeong Telephone No. 82-42-481-5626

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2005/000969

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of :

a. type of material
- [x] a sequence listing
- [ ] table(s) related to the sequence listing

b. format of material
- [ ] on paper
- [x] in electronic form

c. time of filing/furnishing
- [x] contained in the international application as filed
- [x] filed together with the international application in electronic form
- [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2005/000969

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	S. SINHA AND I. LIEBERBURG. "Cellular mechanisms of β-amyloid production and secretion." In: PNAS, (28 September 1999) Vol.96(20):11049-11053, see the whole document.	1-13

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR2005/000969

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US6717031	06.04.2004	US20020104104A1	01.08.2002
		US2004226054A1	11.11.2004

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

LEE, Sang-Yong
4F., Byukcheon Bldg.,
1579-5, Seocho-dong,
Seocho-gu,
Seoul 137-876
Republic of Korea

Date of mailing (day/month/year)
02 March 2006 (02.03.2006)

Applicant's or agent's file reference
5FPO-03-18 PCT 06-005

IMPORTANT NOTIFICATION

International application No.
PCT/KR2005/000969

International filing date (day/month/year)
01 April 2005 (01.04.2005)

1. The following indications appeared on record concerning:

[ ] the applicant [ ] the inventor [X] the agent [ ] the common representative

Name and Address
LEE, Won-Hee
8th Fl. Sung-jl Heights II 642-16
Yoksam-dong Kangnam-ku
Seoul 135-080
Republic of Korea

State of Nationality:
State of Residence:
Telephone No.: 82-2-3453-0507
Facsimile No.: 82-2-3453-8155
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [ ] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address
LEE, Sang-Yong
4F., Byukcheon Bldg.,
1579-5, Seocho-dong,
Seocho-gu,
Seoul 137-876
Republic of Korea

State of Nationality:
State of Residence:
Telephone No.: 82-2-588-5757
Facsimile No.: 82-2-588-5790
Teleprinter No.:

3. Further observations, if necessary:
The appointment of the agent of record has been revoked. A new agent has been appointed, as indicated in Box 2.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[X] the designated Offices concerned
[ ] the elected Offices concerned
[X] other: LEE, Won-Hee

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No. +41 22 338 7090

Authorized officer
Mehdi FILALI
Telephone No. (41-22) 338 8784

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
5FPO-03-18		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/KR2005/000969	**01 APRIL 2005 (01.04.2005)**	01 APRIL 2004 (01.04.2004)

Applicant

EWHA UNIVERSITY-INDUSTRY COLLABORATION FOUNDATION et al

This International search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
      ☒ The international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
   b. ☒ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, see Box No. I.
2. ☐ Certain claims were found unsearchable (See Box No. II)
3. ☐ Unity of invention is lacking (See Box No. III)
4. With regard to the title,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established by this Authority to read as follows:

5. With regard to the abstract,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.
6. With regard to the drawings,
   a. the figure of the drawings to be published with the abstract is Figure No. 1
      ☒ as suggested by the applicant.
      ☐ because the applicant failed to suggest a figure
      ☐ because this figure better characterizes the invention
   b. ☐ none of the figure is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2005/000969

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of :

a. type of material

- [x] a sequence listing
- [ ] table(s) related to the sequence listing

b. format of material

- [ ] on paper
- [x] in electronic form

c. time of filing/furnishing

- [x] contained in the international application as filed
- [x] filed together with the international application in electronic form
- [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2005/000969

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7 C12N 15/63, A01K 67/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean patents and applications for inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKIPASS, Delphion, NCBI PubMed, GenBank database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	R. GERLAI, ET. AL. ""Behavioral impairment of APP(V717F) mice in fear conditioning: is it only cognition?." In: Behav. Brain Res., (November 2002) Vol.136(2):503-509, see the whole document.	1-13
Y	E. M. ROCKENSTEIN, ET. AL. "Levels and Alternative Splicing of Amyloid β Protein Precursor (APP) Transcripts in Brains of APP Transgenic Mice and Humans with Alzheimer's Disease." In: J. Biol. Chem., (24 November 1995) Vol.270(47):28257-28267, see the abstract, figure1, 2, and Materials and Methods.	1-13
Y	B. P. F. RUTTEN, ET. AL. "No alterations of hippocampal neuronal number and synaptic bouton number in a transgenic mouse model expressing the β-cleaved C-terminal APP fragment." In: Neurobiol. Dis., (March 2003) Vol.12(2):110-120, see the abstract and Materials and Methods.	1-13
Y	T. KAWARABAYASHI, ET. AL. "Accumulation of β-Amyloid Fibrils in Pancreas of Transgenic Mice." In: Neurobiol. Aging, (March-April 1996) Vol.17(2):215-222, see the abstract, figure1, and Methods.	1-13
A	US 6717031 B2 (KATE DORA GAMES; DALE BERNARD SCHENK; LISA CLAIRE MCCONLOGUE; PETER ANDREW SEUBERT; RUSSELL E. RYDEL) 6 April 2004 (2004-04-06), see the whole document.	1-13

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 JUNE 2005 (27.06.2005)	27 JUNE 2005 (27.06.2005)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
920 Dunsan-dong, Seo-gu, Daejeon 302-701,
Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

AHN, Kyu Jeong

Telephone No. 82-42-481-5026

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/KR2005/000969

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	S. SINHA AND I. LIEBERBURG, "Cellular mechanisms of β -amyloid production and secretion." In: PNAS, (28 September 1999) Vol.96(20):11049-11053, see the whole document.	1-13

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No
PCT/KR2005/000969

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US06717031	06.04.2004	US20020104104A1	01.08.2002
		US2004226054A1	11.11.2004

PCT/KR2005/000969

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

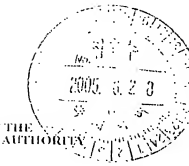
To:
LEE, Won-Hee

8th Fl.Sung-ji Heights Bld42-16 Yoksam-dong Kangnam-ku
Seoul 135-080 Republic of Korea

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43bis.1)



Date of mailing *(day/month/year)*	27 JUNE 2005 (27.06.2005)
Applicant's or agent's file reference 5FPO-03-18	FOR FURTHER ACTION See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/KR2005/000969	**01 APRIL 2005 (01.04.2005)**	01 APRIL 2004 (01.04.2004)

International Patent Classification (IPC) or both national classification and IPC

IPC7 C12N 15/63

Applicant

EWHA UNIVERSITY-INDUSTRY COLLABORATION FOUNDATION et al

1. This opinion contains indications relating to the following items:

- [X] Box No. I Basis of the opinion
- [ ] Box No. II Priority
- [ ] Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- [ ] Box No. IV Lack of unity of invention
- [X] Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- [ ] Box No. VI Certain documents cited
- [ ] Box No. VII Certain defects in the international application
- [ ] Box No. VIII Certain observations on the international application

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.
For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA/KR	Date of completion of this opinion	Authorized officer
Korean Intellectual Property Office 920 Dunsan-dong, Seogu, Daejeon 302-701, Republic of Korea	27 JUNE 2005 (27.06.2005)	AHN, Kyu Jeong
Facsimile No. 82-42-472-7140		Telephone No. 82-42-481-5024

Form PCT/ISA/237 (cover sheet) (April 2005)

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No
PCT/KR2005/000969

Box No. I Basis of this opinion

1 With regard to the language, this opinion has been established on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ This opinion has been established on the basis of a translation from the original language into the following language _____, which is the language of a translation furnished for the purposes of international search (under Rules 12.3 and 23.1(b)).

2. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:

a. type of material
- ☒ a sequence listing
- ☐ table(s) related to the sequence listing

b. format of material
- ☐ on paper
- ☒ in electronic form

c. time of filing/furnishing
- ☒ contained in the international application as filed.
- ☒ filed together with the international application in electronic form.
- ☐ furnished subsequently to this Authority for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additioanl copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/KR2005/000969

Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-13	YES
	Claims	None	NO
Inventive step (IS)	Claims	None	YES
	Claims	1-13	NO
Industrial applicability (IA)	Claims	1-13	YES
	Claims	None	NO

2. Citations and explanations :

The following documents have been considered for the purpose of this written opinion:

D1: Behav. Brain Res., Vol.136(2):503-509
D2: J. Biol. Chem., Vol.270(47):28257-28267
D3: Neurobiol. Dis., Vol.12(2):110-120
D4: Neurobiol. Aging, Vol.17(2):215-222

1. Novelty and Inventive Step

The present invention relates to a transgenic animal having Alzheimer's disease. Particularly, the subject matter of claims 1 to 11 relates to a vector for inducing Alzheimer's disease in animal model, containing a carboxyl-terminal fragment of human amyloid precursor (hAPP) which contains mutation V717F(βCTF99(V717F)). The subject matter of claims 11 to 13 relates to a transgenic mouse having induced Alzheimer's disease pathology generated by microinjection of the said vector into a pronuclei of a fertilized oocyte followed by generating mice.

D1 discloses a transgenic mouse exhibiting learning and memory performance deficits, and altered emotionality, which overexpresses hAPP carrying the mutation V717F.

D2 discloses a transgene comprising a platelet-derived growth factor promoter, APP carrying the mutation V717F (APPInd), intron, SV40 pA region.

D3 discloses transgenic mice expressing human β-CTF with the I45F mutation under the control of the prion protein promoter.

D4 discloses transgenic mice expressing human β-CTF, which are generated using the transgene of signal peptide and C-terminal 99 residues of APP under the control of CMV enhancer/chicken β-actin promoter.

(Continued on Supplemental Box.)

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/KR2005/000969

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of :

Box V. 2

None of the prior art documents D1 to D4 disclose the vector containing only a C-terminal fragment of hAPP carrying V717F mutation (βCTF99V717F), and a transgenic mouse generated by the same.

Thus, the novelty of the subject matter of claims 1 to 13 can be acknowledged [PCT Article 33(2)].

Since there are transgenic mice expressing β-CTF or β-CTF(I45F) to address the potential neurotoxicity of the β-CTF of hAPP (D3, D4), and D1, D2 disclose that V717F mutation of APP alters proteolytic processing of APP and the mouse expressing APP(V717F) exhibits Alzheimer's disease pathology, it appears to be obvious to a person skilled in the art to generate transgenic mice expressing β-CTF carrying V717F mutation from the teachings of D1 to D4.

The claims 1-13 therefore cannot be regarded as meeting the requirement of inventive step [PCT Article 33(3)].

2. Industrial Applicability

The subject matter of claims 1-13 is considered to be industrially applicable [PCT Article 33(4)].